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(71) Applicant *(for all designated States except US)*: **ERASMUS UNIVERSITEIT ROTTERDAM** [NL/NL]; Dr. Molewaterplein 50, NL-3015 GE Rotterdam (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **VERSTEGEN, Monique, Maria, Andrea** [NL/NL]; Vondellaan 41, NL-2902 AR Capelle a/d IJssel (NL). **WOGNUM, Albertus, Wernerus** [NL/NL]; 1e Jerichostraat 47D, NL-3061 GC Rotterdam (NL). **WAGEMAKER, Gerard** [NL/NL]; Adrianalaan 138, NL-3053 ME Rotterdam (NL).

(74) Agent: **PRINS, A., W.**; Vereenigde, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

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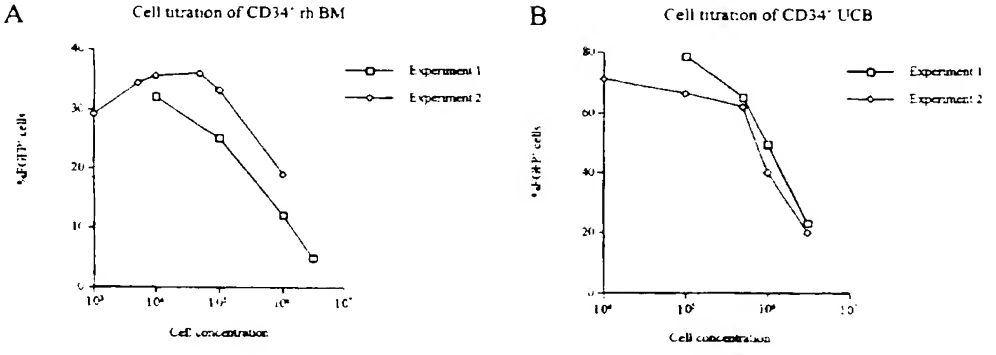
(54) Title: IMPROVED METHODS AND MEANS FOR RETROVIRAL GENE DELIVERY



(57) Abstract: The invention provides methods, compositions and uses of said compositions for transducing target cells with gene delivery vehicles of retroviral origin comprising providing a selection of CD34 positive cells from a culture of cells, taking a plurality of samples of said CD34 positive population, diluting and/or concentrating said samples to provide a range of concentrations of cells per volume, contacting samples in said range with a composition comprising said gene delivery vehicles and determining the optimal concentration of target cells for efficient transduction and diluting or concentrating said CD34 positive population to said optimal concentration and contacting said population with said gene delivery vehicles to allow for said transduction of target cells.

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Title: Improved methods and means for retroviral gene delivery

The present invention relates to the field of recombinant retroviral particles, to retroviral gene delivery vehicles. To methods for producing particles and vehicles as well as uses of the particles or vehicles in improved transduction methods, compositions for transduction as well as pharmaceutical compositions for the treatment of disorders with a genetic component. In particular the invention provides in one of its embodiments a method of gene transfer into e.g. pluripotent hematopoietic stem cells and their descendants, enabling successful transduction of 90% of CD34+ cells, including transplantable cell populations comprising hematopoietic stem cells that give rise to progeny expressing the transduced gene(s). The use of the method e.g. includes and is included in a method for treatment of a variety of hereditary and acquired human diseases by transfer of therapeutically active genes into hematopoietic stem cells and genetic marking of suchs cells.

The hematopoietic system produces perpetually large numbers of blood cells, which have a limited life span and need to be perpetually renewed throughout the life of a mammal. This renewal is maintained through proliferation and differentiation of a small number of hematopoietic stem cells in the bone marrow. The definition of stem cells is not always clear within the art. Herein a functional definition is used, which defines stem cells as those cells capable of (long term) reconstitution of a hematopoietic system. This definition is often felt to include at least some early progenitor cells. Since blood cells virtually reach every organ, hematopoietic stem cells are a highly suitable target for gene therapy for a variety of hereditary and acquired diseases within and outside the hematopoietic system. Unfortunately, retrovirus mediated gene transfer has met with only limited success due to the difficulty of obtaining sufficient numbers of successfully transduced,

transplantable, long-term repopulating hematopoietic stem cells.

Recent advances in understanding stem cell biology include the discovery of heterogeneity of stem cells both in terms of maturity as well as the discovery of novel growth factors thought to control their proliferation and differentiation and the possibility to purify hematopoietic stem cells by selection for the CD34 surface marker. Retrovirus mediated gene transfer has been greatly benefited by co-localization of cells and virus using a recombinant fibronectin fragment, whereas the importance of selecting a suitable retrovirus receptor has been recognized. Based on these advances, we have analyzed the variables influencing gene transfer during the transduction procedure and selected a highly efficient producer cell subclone. With our invention we have made the observation that several binding steps involving receptors and ligands on virus, cells and mediating adherence molecules are needed for successful transduction, each with their specific affinities. Hence, our invention discloses the finding that the transduction procedure should be highly dependent on the relative concentration of these molecules during the tranduction procedure.

Thus the invention provides a method for transducing target cells with gene delivery vehicles of retroviral origin comprising providing a selection of CD34 positive cells from a culture of cells, taking a plurality of samples of said CD34 positive population, diluting and/or concentrating said samples to provide a range of concentrations of cells per volume, contacting samples in said range with a composition comprising said gene delivery vehicles and determining the optimal concentration of target cells for efficient transduction and diluting or concentrating said CD34 positive population to said optimal concentration and contacting said population with said gene delivery vehicles to allow for said transduction of target cells.

As stated herein before, the population of CD34 positive

cells such as they can be found in e.g. umbilical cord blood or bone marrow includes the stem cells as defined herein above (i.e. the cells capable of long term repopulation). We have found that a very important variable in the efficiency of transduction is a ratio between number of cells (or cell concentration) and the number of transducing particles. According to our invention these should be optimized vis a vis one another, which may sometimes lead to increasing particle titers and/or target cell concentrations, but surprisingly also to decreasing viral particles and/or lowering cell concentrations. Therefore a range of concentrations should be tried with samples from the target population of cells. Gene delivery vehicles of retroviral origin are all vehicles comprising genetic material and/or proteinaceous material derived from retroviruses. Typically the most important features of such vehicles are the integration of their genetic material into the genome of a target cell and their capability to transduce stem cells. These elements are deemed essential in a functional manner, meaning that the sequences need not be identical to retroviral sequences as long as the essential functions are present. The methods of the invention are however especially suitable for recombinant retroviral particles, which have most if not all of the replication and reproduction features of a retrovirus, typically in combination with a producer cell having some complementing elements. Normally the retroviral particles making up the gene delivery vehicle are replication defective on their own. The invention is particularly suited for the production of gene delivery vehicles, however other retroviral particles can also be produced according to the invention.

In another embodiment according to the invention not only the concentration of target cells is optimized, but also the concentration of virus. As stated before, optimization of all concentrations involved in binding or interaction is preferred. In order to be able to modify virus titers high

initial titers are preferred. Methods to arrive at those are also provided by the present invention. Thus the invention further provides a method wherein target cell concentrations are optimized further comprising optimizing the concentration of said gene delivery vehicles for optimal transduction efficiency. It is of course that a gene delivery vehicle is intended to read on any vehicle capable of delivering genetic material to a target cell, whether the genetic material is actually a gene, an antisense molecule or a cosuppressive nucleic acid (encoding molecule), etc. Useful nucleic acids to be provided to target cells, e.g. stem cells are well known in the art and include such molecules as to replace inborn errors/deficiencies of the hematopoietic system, which may include hemoglobin genes and their regulatory elements for the thalassemia's and sickle cell anemia's and sequences to repair the various forms of severe combined immunodeficiency, such as caused by adenosine deaminase deficiency and that known as severe X linked immunodeficiency, or genes encoding enzymes for diseases known as lysosomal storage diseases, such as Hurler's, Hunter's, Krabbe's and in particular Gaucher's disease and metachromatic leukodystrophy, or by introducing sequences that confer resistance of the progeny of hematopoietic stem cells to infectious agents, such as HIV, as well as the introduction of suicide genes for cancer therapy and marker genes to track the progeny of transplanted normal and/or malignant hematopoietic stem cells. Another factor involved in binding and/or interaction is a matrix binding both virus and target cell exemplified herein by fibronectin and retronectin. The optimization of their concentration is also part of the present invention. Thus the invention also provides a method as described above wherein said target cells are cultured in the presence of fibronectin, retronectin or a functional equivalent thereof, preferably further comprising optimizing the concentration of said fibronectin, retronectin or said functional equivalent for

optimal transduction efficiency.

Typically the target cells of the present invention comprise populations of CD34 positive cells, which are efficiently transduced by retroviral particles, preferred are those populations wherein said CD34 positive cells comprise umbilical cord blood cells or bone marrow cells. As stated above it is preferred to use high viral titers to be able to optimize all relevant concentrations vis a vis all binding and/or interaction steps. Therefore the invention also provides a method as described above wherein a composition of retroviral gene delivery vehicles of improved titer is applied. According to the invention preferably a method for improving the virus titer is applied which involves improving the producer cell line. Thus the invention also provides a method wherein said virus titer is improved by providing a culture of producer cells of a retroviral gene delivery vehicle, subcloning said culture of producer cells, culturing the resulting subclones and selecting the clones producing the highest virus titers, possibly based on multiple copies of the provirus due to reinfection. Apparently a number of cells from established producer cell lines loose some of their ability to produce effective retroviral particles. Subcloning appears to be a way to select for those cells retaining that ability. Other ways of selecting for such cells are also included in the present invention.

Another factor promoting the transduction efficiency is prior cryopreservation of the CD34+ cells prior to use in the transduction procedure. Typically, umbilical cord blood harvests are cryopreserved prior to use, whereas rhesus monkey cells are predominantly used immediately after procurement, ether or not following stimulation or mobilization of the CD34+ by administration of G-CSF to the animals. Cryopreservation resulted in less variability of the obtained transduction frequencies and, in general, a much higher level of transduction. The mechanisms involved are not clear and may be related to the use of DMSO, damage to the

cell membrane, more prominent availability of the GALV-receptor protein and/or upregulation of the receptor gene expression. Therefore, in one embodiment a harvest of stem cells is frozen, optionally stored, and thawed prior to performing a transduction method of the invention.

The method of improving virus titers can also be used apart from the improvement of transduction. Thus the invention also provides a method for producing retroviral particles at high titers, comprising providing a culture of producer cells producing retroviral particles, subcloning said culture of producer cells, culturing the resulting subclones and selecting the cultures producing the highest virus titers. Again the invention is preferably applied to gene delivery vehicle production. Thus the invention provides in yet another embodiment a method as just described wherein said retroviral particles are gene delivery vehicles. Typically producer cells are well known in the art. The preferred ones are mouse fibroblast cells, originating from PG13 which is pseudotyped with the gibbon ape leukemia virus (GALV). GALV-receptors (GLV-1 or Pit-1) are present on human hematopoietic cells.

The invention also includes compositions obtainable by the methods of the invention. Thus included are compositions comprising retroviral particles at high titer obtainable by a method as disclosed above, preferably those wherein said retroviral particles are gene delivery vehicles. Preferably such a composition comprises retroviral particles capable of transducing hematopoietic stem cells and/or progenitor cells, preferably wherein said retroviral particles are capable of transducing umbilical cord blood cells and/or bone marrow cells.

The invention also provides the pharmaceutical use of these compositions, particularly in the treatment of diseases having a genetic component, such as the various genetic hemoglobin orders, the large group of rare diseases collectively known as severe combined immune deficiencies,

the group of lysosomal storage diseases, especially with a strong hematopoietic and/or visceral expression, such as Gaucher's disease, but also possibly Krabbe's disease, as well as in the treatment of infectious disease, notably HIV infection, or cancer. Typically the use of a composition comprising retroviral particles will involve the transduction of CD34 positive target cells. Such transduced cells are typically made in vitro and are also part of the present invention. Thus the invention provides a composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells transduced with a composition of retroviral particles according to the invention, or a composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells, said composition being obtainable by a method according to the invention.

Detailed description

The object of the present invention is to provide a generally applicable method for retrovirus mediated transfer of therapeutic and marker genes into pluripotent hematopoietic stem cells.

The invention includes the unexpected and surprising finding that transduction is dependent on the concentration of target cells during the transduction period and that selection of high titer subclones of a by itself effective producer cell line decreases this dependence on cell concentration. These two findings enable a reproducible, highly efficient method of gene transfer into hematopoietic stem cells, which maintain their transplantability and provide descendant containing the gene of interest and expressing this gene following transplantation in recipient

subjects. Proof of principle will be obtained using transplantation of successfully marked CD34+ cells into irradiated nonhuman primates. For human umbilical cord blood stem cells, we have obtained proof of principle by transplantation of human umbilical cord blood stem cells in immunodeficient NOD/SCID recipients. To test for transduction of transplantable human multilineage and/or pluripotent hemaopoietic stem cells, EGFP transduced CD34+ cells were transplanted into immunodeficient mice irradiated with 3.5 Gy (g-rays total body irradiation. The mice were sacrificed at day 35 to measure content of repopulating cells and to assess the multilineage nature of the transduced cells by flow cytometry. Typically, the optimized procedure resulted in percentages of up to 80% EGFP expression (Table 1) which was multilineage in nature (Figure 4). Since only a small subset of CD34$^+$ cells has the capacity to produce offspring after transplantation it was thought to be of interest to relate EGFP expression frequencies of CD45$^+$ cells in the engrafted NOD/SCID mice with the frequency of EGFP$^+$ cells in the transplanted CD34$^+$ cells (Figure 5). This analysis revealed a threshold of transduction of repopulating cells relative to the CD34$^+$ cells, which explains why substantial transduction frequencies in CD34$^+$ cells do not necessarily result in similar frequencies following transplantation, and also demonstrated that with the optimized procedure up to 80% of the multilineage repopulating cells should express the transgene.

For human umbilical cord blood stem cells, provisional proof of principle has already been obtained (Van Hennik et al., Blood, 1998) by transplantation of transduced stem cells into immunodeficient NOD/SCID recipients under conditions resulting in a lower transduction frequency of CD34+ than has become possible by the present invention. Hence, clinical application of the method for autologous umbilical cord blood gene therapy for a variety of diseases has become a feasible option for therapy by the present invention.

Materials and Methods

Viral vector and packaging cell line

The pseudotyped retroviral producer cell line PG13/EGFP7 was kindly provided by J. Barquinero (Institut de Recerca Oncologica, Barcelona, Spain). The cell line was developed by transducing the PG13 packaging cell line (kindly provided by D. Miller, Fred Hutchinson Cancer Research Centre, Seattle, WA) with 0,45 μm filtered supernatant from PA317/EGFP cell cultures. (Limon A et al., (1997), Blood, 90:3316-21 21). EGFP expression was analyzed by flow cytometry and bright single cells were sorted on 96-well plates by using an EPICS Elite ESP flow cytometer coupled to an autoclone device (both from Coulter, Miami, FL, USA). Single clones were cultured as previously described. (Limon A et al., (1997), Blood, 90: 3316-21). The PG13/EGFP7 cell line was subcloned by diluting the cells to 1 cell per well of a 96-well plate. Single subclones were cultured as described and analyzed for transduction efficiency on rhesus BM en UCB CD34+ cells. The viral titer of the cell line (original and subclones) was in the order of 10^5 - 10^7 infectious particles per ml as determined by supernatant titration on cultured human HeLa cells and Rat-2 cells. Absence of replication-competent virus was verified by failure to transfer GFP-expression from a transduced cell population to a secondary population.

Subcloning of the PG13 SF-EGFP packaging cell line/vector combination (Figure 2

Subcloning of the PG13/SF-EGFP7 virus producer cell line was performed using limiting dilution to grow one cell per well of a 96-well plate in culture medium consisting of an enriched version of Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD ; Merchav S et al. 1994 , Int J Cell Cloning, 2: 356-67 ; Wagemaker G et al. 1981 , Cell

Tissue Kinet, 13: 505-17). Supplemented with 10% FCS. Growing clones were harvested and grown in T75cm² flasks until 80% confluency and subsequently tested for transduction efficiency.

Determination of the virus titer (Figure 2)

To determine the virus titer of all clones, diluted supernatants of these clones were used to transduce cells of the Rat-2 cell line and HeLa cell line. The producer cell lines (PG13/SF-EGFP7, clone 1, clone 2, clone 3 and clone 5) were grown in T75 cm² culture flasks until 80% confluency as described above. Subsequently, 2000 Rat-2 cells and HeLa cells were cultured for 24 hours in dilutions of 0,45 μm filtered virus supernatant of the different clones of the virus producer cell line in 12 wells of a 24-wells plate. As a control, 1 well did not contain virus supernatant but culture medium solely. The virus supernatant was removed and substituted with fresh culture medium. The transduced cells were harvested at confluency and the transduction efficiency was determined by flow cytometry (FACSCalibur, Becton & Dickinson). The virus titer was determined by calculating the number of cells initially cultured (2000) that were transduced at a certain dilution of the virus supernatant.

Retroviral transduction

Supernatants containing recombinant retrovirus were generated by culturing approximately 80% confluent producer cells for 12 hours in culture medium consisting of serum-free enriched version of Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg, MD) (Merchav S et al. (1984), Int J Cell Cloning, 2: 356-67; Wagemaker et al. 1980, Cell Tissue Kinet, 13: 505-17). Media for all cultures routinely included 100 U/ml of penicillin and 100 μg ml of streptomycin. The cultures were maintained at 37°C with 10% CO_2 (measured every 15' with read-outs between 9,5% and 11%) in a humidified atmosphere. The culture supernatant was

subsequently produced and passed through a 0,45 µm filter. To enhance the transduction efficiency, Falcon 1008 (35 mm) bacteriological culture dishes were coated with the recombinant fibronectin fragment CH-296 (Takara Shuzo, Otsu, Japan) at a concentration of 10 µg/cm² as described previously. (Moritz T et al. (1996), Blood, 88:855-62). CD34-selected UCB, human BM, normal rhBM or rhBM from G-CSF and/or Flt3-L treated monkeys were prestimulated for 2 days in enriched Dulbecco's medium with combinations of the human recombinant growth factors Flt3-L (50 ng/ml, kindly provided by Amgen, Thousand Oaks, CA, USA), thromopoietin (huTPO and rhTPO, 10 ng/ml, kindly provided by Genentech, South San Francisco, CA, USA) and stem cell factor (SCF, 100 ng/ml). Before adding purified subsets to the fibronectin-coated dishes, the CH-296 fibronectin fragment was preincubated with supernatant containing the pseudotyped vector for 1 hour at 37°C. (Moritz et al. (1996), Blood, 88: 855-62). (Hanenberg H et al. (1996), Nat Med, 2: 876-82). Subsequently, nucleated cells were resuspended in the vector-containing supernatant supplemented with hematopoietic growth factors (HGF), and added to the dishes. Over a period of 2 days, culture supernatant was replaced completely by resuspending nonadherent cells into fresh retrovirus supernatant and HGF. Finally, the cells were harvested and analysed by flow cytometry and cell cycle analysis.

All umbilical cord blood samples used were cryopreserved prior to use, as were the indicated samples of rhesus monkey bone marrow. For cryopreservation, the cells were suspended in HEPES buffered Hanks' balanced salt solution, supplemented with 22,5% foetal calf serum and 7,5% DMSO in a concentration ranging from 20-200 x 10⁶/ml. The cells were frozen in ampoules of 1, 1,5 or 5 ml volume using a Planer Biomed Kryo 10 controlled cryopreservation machine during the crystallization at a rate of -1°C per minute. Prior to use, cryopreserved cells were thawed by the standard so called "step-wise dilution" method, thoroughly washed and

resuspended in the medium used for transduction.

Target cell titrations (Figure 1)

Rhesus monkey BM (rh BM) and human umbilical cord blood (UCB) cells were titrated from $3x10^5$ to 10^3/ml during the transduction assay. The producer cell line was, as standard, cultured in T75 cm^2 flasks filled with 10 ml serumfree medium as described above until 80% confluency. During the transduction, the virus supernatant was refreshed once. After 2 days prestimulation and 2 days of supernatant infection the cells were harvested and the transduction efficiency was determined by flow cytometry.

Transplantation of transduced human UCB cells into NOD/SCID mice

Male, specified pathogen-free (SPF) NOD/LtSz-scid/scid mice (NOD/SCID) were bred and housed under SPF conditions and supplied with sterile food and drinking water containing 100 mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) *ad libitum*. Housing, care and all animal experimentation were done in conformity with legal regulations in The Netherlands, which include approval by a local ethical committee. All mice received total body irradiation (TBI) at 3.5 Gy, delivered by a ^{137}Cs source adapted for the irradiation of mice (Gammacell, Atomic Energy of Canada, Ottawa, Canada), 2-4 hours before transplantation. The transplants were suspended in 200 µl H+H and injected i.v. into a lateral tail vein. Transplanted cell numbers were 2x10^5 CD34$^+$ UCB cells. At day 35 after transplantation, the mice were killed by CO_2 inhalation followed by cervical dislocation, both femurs and the spleen were isolated and BM suspensions were prepared by flushing. After counting, BM cells were analyzed by flow cytometry to determine the percentage human EGFP$^+$ cells in the mouse BM and their multilineage nature determined by flow cytometry. Data were expressed as median (range). Statistical comparisons were performed according to Mann Whitney U-test.

Two tailed P values of <0.05, were considered significant. Actual significance levels are indicated in table 1 and in the figures.

Transplantation assays in rhesus monkeys

To test for transduction of transplantable multilineage and/or pluripotent hematopoietic stem cells, EGFP-transduced CD34+ cells are transplanted into rhesus monkeys subjected to 9 Gy (6 MV X-rays) total body irradiation in cell numbers range from hundred thousand to several millions of CD34+ cells per kg body weight, either immediately following transduction or selected for expression of the EGFP gene by cell sorting using a FACS Vantage cell sorter (Becton Dickinson). The transplantation procedure has been described in detail (Neelis KJ et al. (1997), Exp Hematol, 25:1094-103). The monkeys are followed daily for expression of EGFP in peripheral blood subsets, and weekly for expression in bone marrow subsets, using flow cytometric surface marker labeling to identify the different blood cell lineages. As indicated in figure 3 the transduction of stem cells using methods of the invention can be reproducibly very high.

Brief description of the drawings

Figure 1
Cell titration of CD34-selected rhesus BM cells (A) and human umbilical cord blood (UCB) cells. (B) during infection with the GALV-pseudotyped PG13/SF-EGFP7 retroviral packaging cell line/vector combination. The highest levels of EGFP$^+$ cells were found after transduction of $5x10^4$/ml rh BM cells or 10^5/ml UCB cells with 35% and 80%, respectively.

Figure 2
Subcloning of the PG13/SF-EGFP7 packaging cell line/vector combination resulted in clones with different virus titers as determined by supernatant dilution on Rat-2 cells (A) and HeLa cells (B). Clone 1 and 2 (PG13/SF-EGFP7.1 and PG13/SF-EGFP7-2) showed the highest virus titers, whereas clone 3 and 5 (PG13/SF-EGFP7.3 and PG13/SF-EGFP7.5) resulted in low virus titers. Transduction of $5x10^5$ rh BM cells with PG13/SF-EGFP7 and clones 1-5, 2b, 4b and 5b (C) resulted in levels of EGFP transduced rh BM cells that correlates with the virus titers. Clone 1, 2 and 2b showed EGFP levels of 80%-90% which was higher as compared to the parental PG13/SF-EGFP7 producer. Subclones of clone 1 and 5 (PG13/SF-EGFP7.1.2 and PG13/SF-EGFP7.5.1, respectively) resulted in similar transduction levels as the parental cell lines. Decreasing cell numbers (from 10^6 tot 10^5 cells/ml during transduction using virus supernatant of PG13/SF-EGFP7 resulted in increasing transduction efficiency ranging from 10% to 40% (D). Transduction with supernatant from subclone PG13/SF-EGFP7.1 resulted in higher efficiencies (80% to 90% EGFP$^+$ cells) without the titration effect caused by increasing cell numbers/ml.

Figure 3
The effect of prior cryopreservation on transduction

efficiency. Briefly, rhesus monkeys were treated with 100 μg/kg G-CSF for 4 consecutive days, after which bone marrow was procured and used in the described transduction procedure after selection and isolation of cells expressing CD34. The figure shows the transduction frequencies obtained for bone marrow samples immediately used ("fresh") or cryopreserved ("frozen") in comparison to bone marrow from rhesus monkeys taken from a rhesus monkey bone marrow bank ("steady state rhBM"). The differences between the "frozen" and the "fresh" cells is statistically significant ($p=0.01$).

Figure 4
Chimerism and EGFP expression levels in a chimeric NOD/SCID mouse BM 35 days after transplantation of 10^5 $CD34^+$ UCB cells of which 93% expressed the EGFP gene. The bright green autofluorescence on the X-axes vs CD45 on the Y-axes clearly shows that almost all human ($CD45^+$) cells (80%) express the EGFP. The right panel shows the distribution of $EGFP^+$ (❑) and $EGFP^-$ cells (■) in all hematopoietic lineages assessed.

Figure 5
Percentage of EGFP positive $CD45^+$ cells in NOD/SCID mice 35 days after transplantation related to the percentage of primary EGFP positive $CD34^+$ cells transplanted. (●) AM12/MFG-EGFP transductions; (○) PG13/SF-EGFP transductions. The regression line of the data without the amphotropic transductions is identical to that shown of all data pooled.

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CLAIMS

1. A method for transducing target cells with gene delivery vehicles of retroviral origin comprising providing a selection of CD34 positive cells from a culture of cells, taking a plurality of samples of said CD34 positive population, diluting and/or concentrating said samples to provide a range of concentrations of cells per volume, contacting samples in said range with a composition comprising said gene delivery vehicles and determining the optimal concentration of target cells for efficient transduction and diluting or concentrating said CD34 positive population to said optimal concentration and contacting said population with said gene delivery vehicles to allow for said transduction of target cells.

2. A method according to claim 1 further comprising optimizing the concentration of said gene delivery vehicles for optimal transduction efficiency.

3. A method according to claim 1 or 2 wherein said target cells are cultured in the presence of fibronectin, retronectin or a functional equivalent thereof.

4. A method according to claim 3 further comprising optimizing the concentration of said fibronectin, retronectin or said functional equivalent for optimal transduction efficiency.

5. A method according to any one of claims 1-4, wherein said CD34 positive cells comprise umbilical cord blood cells or bone marrow cells.

6. A method according to any one of the afore going claims wherein composition of retroviral gene delivery vehicles of improved titer is applied.

7. A method according to claim 6 wherein said virus titer is improved by providing a culture of producer cells of a retroviral gene delivery vehicle, subcloning said culture

of producer cells, culturing the resulting subclones and selecting the cultures producing the highest virus titers.

8. A method for producing retroviral particles at high titers, comprising providing a culture of producer cells producing retroviral particles, subcloning said culture of producer cells, culturing the resulting subclones and selecting the cultures producing the highest virus titers.

9. A method according to claim 8, wherein said retroviral particles are gene delivery vehicles.

10. A method according to any one of claims 7-9 wherein said producer cells are cells of hematopoietic origin.

11. A method according to claim 10 wherein said producer cells originate from PG13.

12. A composition comprising retroviral particles at high titer obtainable by a method according to claim 8.

13. A composition according to claim 12 wherein said retroviral particles are gene delivery vehicles.

14. A composition according to claim 12 or 13 wherein said retroviral particles are capable of transducing hematopoietic stem cells and/or progenitor cells.

15. A composition according to any one of claims 12-14 wherein said retroviral particles are capable of transducing umbilical cord blood cells and/or bone marrow cells.

16. A composition according to any one of claims 12-15 for use as a pharmaceutical.

17. Use of a composition according to any one of claims 12-15 in the transduction of CD34 positive target cells.

18. A composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of CD34 positive cells transduced with a composition according to any one of claims 12-15.

19. A composition for the treatment of a hereditary disease or a pathological condition related to a genetic defect or a genetic aberration, comprising a plurality of

CD34 positive cells, said composition being obtainable by a method according to any one of claims 1-11.

20. Use of a composition according to claim 18 or 19 in the preparation of a medicament for the treatment of the various genetic hemoglobin orders, the large group of rare diseases collectively known as severe combined immune deficiencies, the group of lysosomal storage diseases, especially with a strong hematopoietic and/or visceral expression, such as Gaucher's disease, but also possibly Krabbe's disease, as well as in the treatment of infectious disease, notably HIV infection, or cancer.

Repopulation of EGFP-transduced $CD34^+$ UCB cells in NOD/SCID mice

Virus producer	Transduction efficiency %EGFP	$EGFP^+$ /chimeric mice	Chimerism in NOD/SCID %CD45	$\%EGFP^+$ in $CD45^+$ cells ± SD (range)
PG13/SF-EGFP7	66	4/4	8 (3-12)	23 ± 17 (2–41)
PG13/SF-EGFP7.1	85	5/5	3 (1-6)	63 ± 17 (38–80) *

* $p<0.009$

Table 1

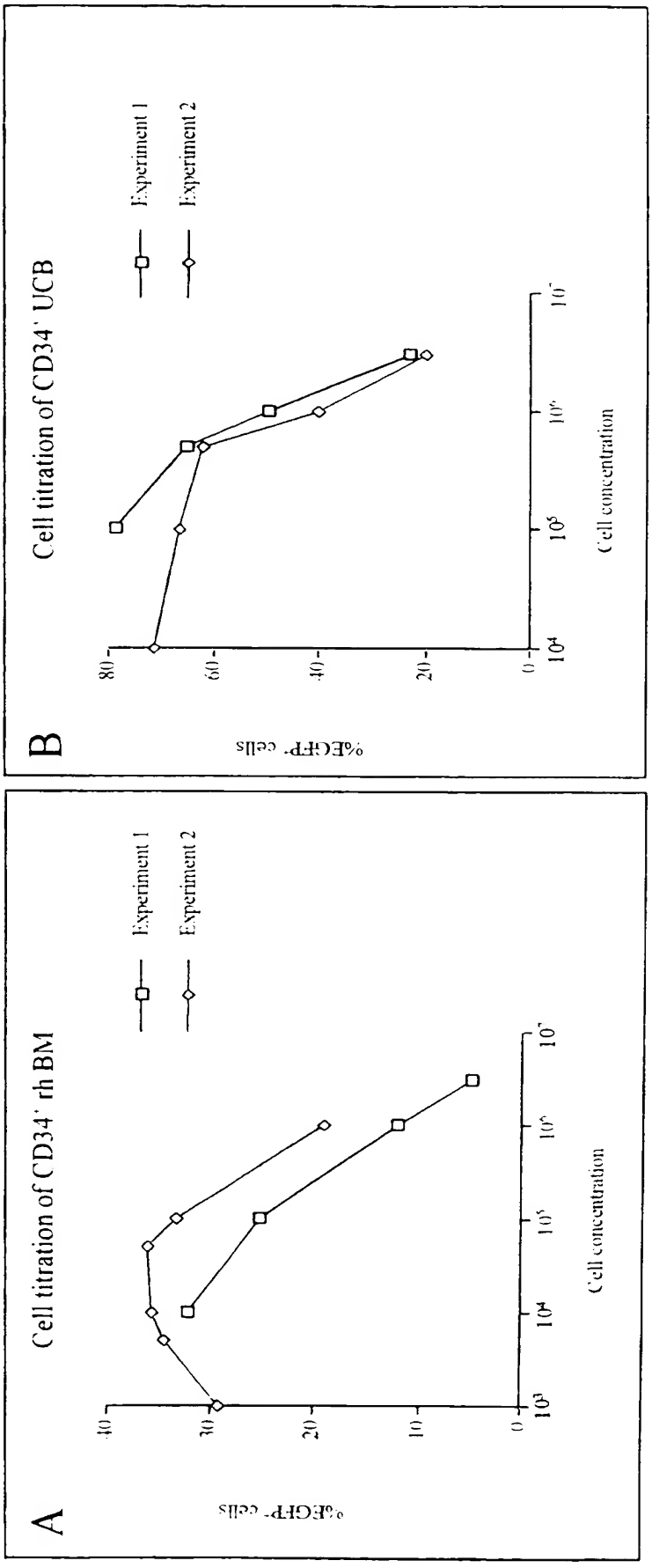
A
Cell titration of CD34+ rh BM
Experiment 1
Experiment 2
%EGFP+ cells
Cell concentration
B
Cell titration of CD34+ UCB
Experiment 1
Experiment 2
%EGFP+ cells
Cell concentration

Fig. 1

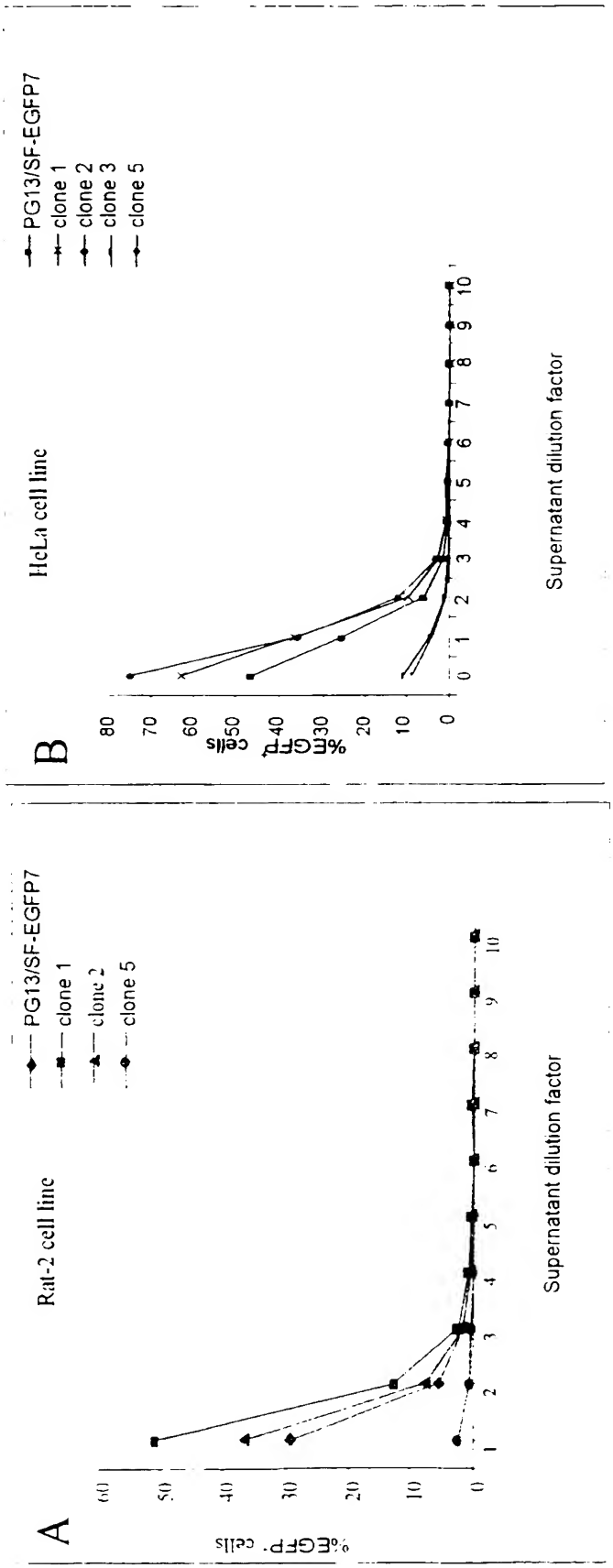
B
HeLa cell line
PG13/SF-EGFP7
clone 1
clone 2
clone 3
clone 5
%EGFP cells
Supernatant dilution factor
A
Rat-2 cell line
PG13/SF-EGFP7
clone 1
clone 2
clone 5
%EGFP+ cells
Supernatant dilution factor

Fig. 2

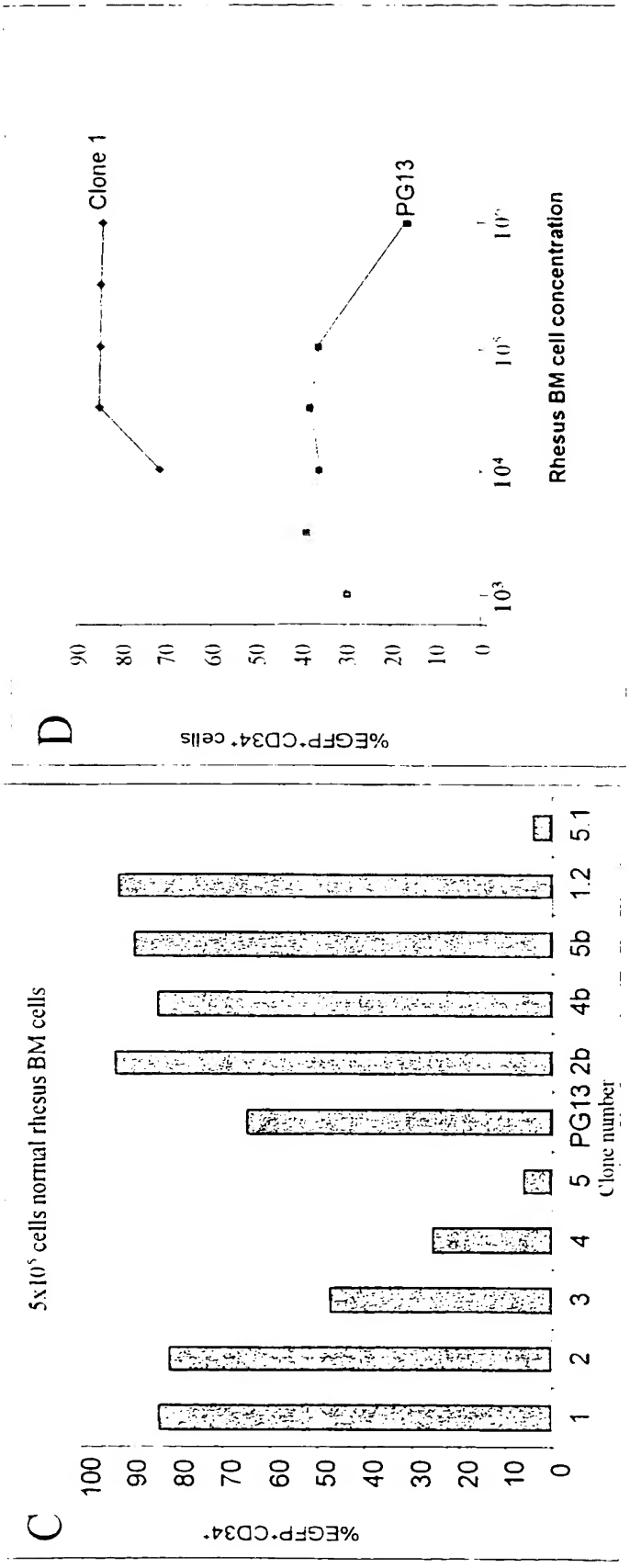
D
%EGFP+CD34+ cells
90
80
70
60
50
40
30
20
10
0
Clone 1
PG13
10^3
10^4
Rhesus BM cell concentration
C
%EGFP+CD34+
100
90
80
70
60
50
40
30
20
10
0
5x10^5 cells normal rhesus BM cells
1
2
3
4
5
PG13
2b
4b
5b
1.2
5.1
Clone number

Fig. 2 (cont.)

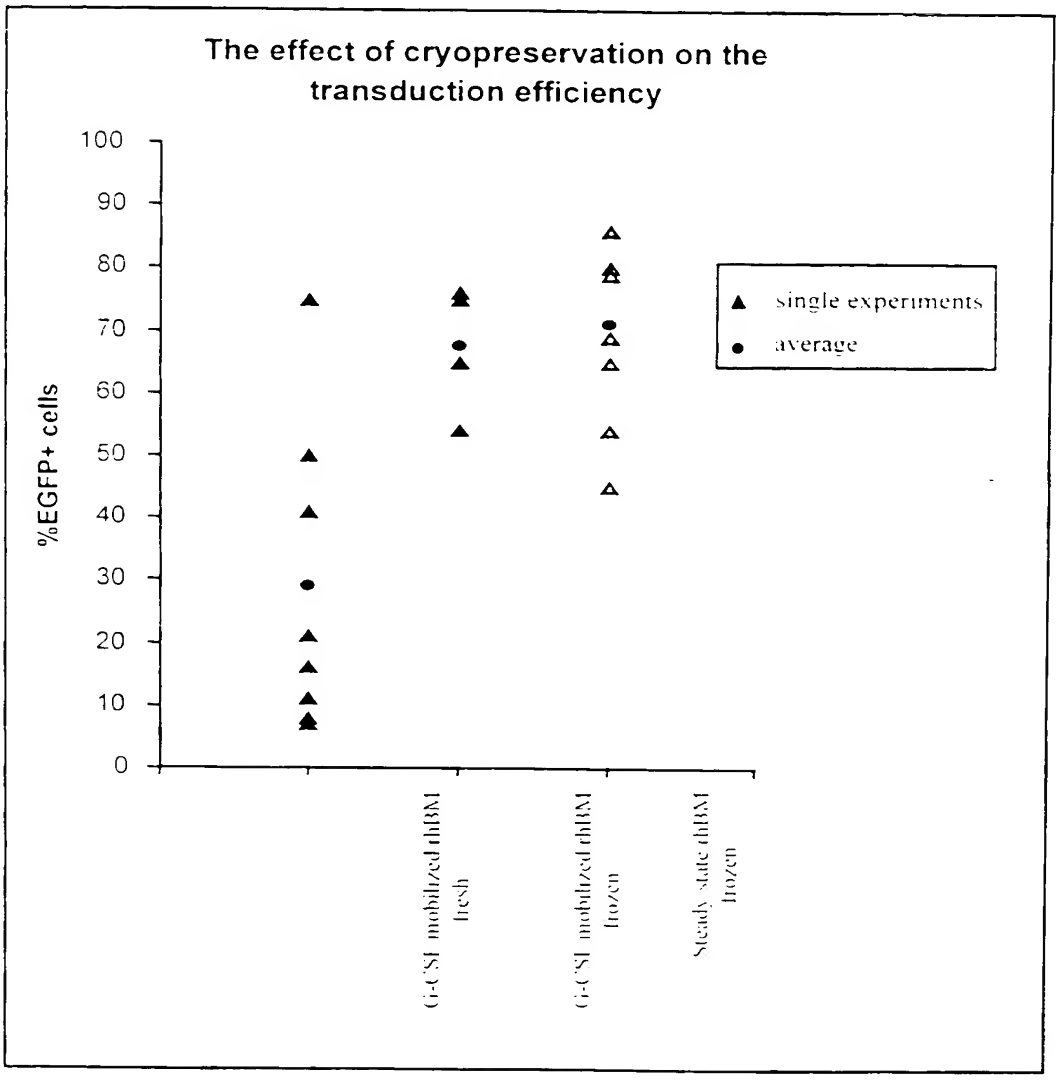
The effect of cryopreservation on the transduction efficiency
%EGFP+ cells
100
90
80
70
60
50
40
30
20
10
0
single experiments
average
G-CSF mobilized rhBM fresh
G-CSF mobilized rhBM frozen
Steady state rhBM frozen

Fig. 3

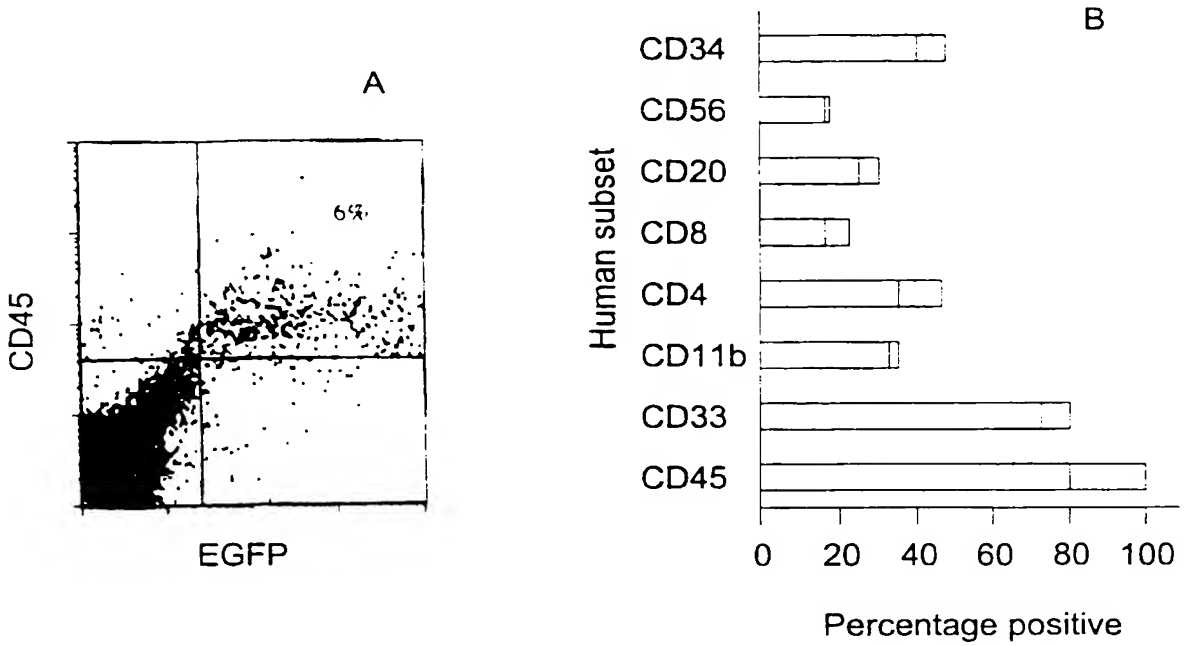
A
CD45
EGFP
B
CD34
CD56
CD20
CD8
CD4
CD11b
CD33
CD45
Human subset
0
20
40
60
80
100
Percentage positive

Fig. 4

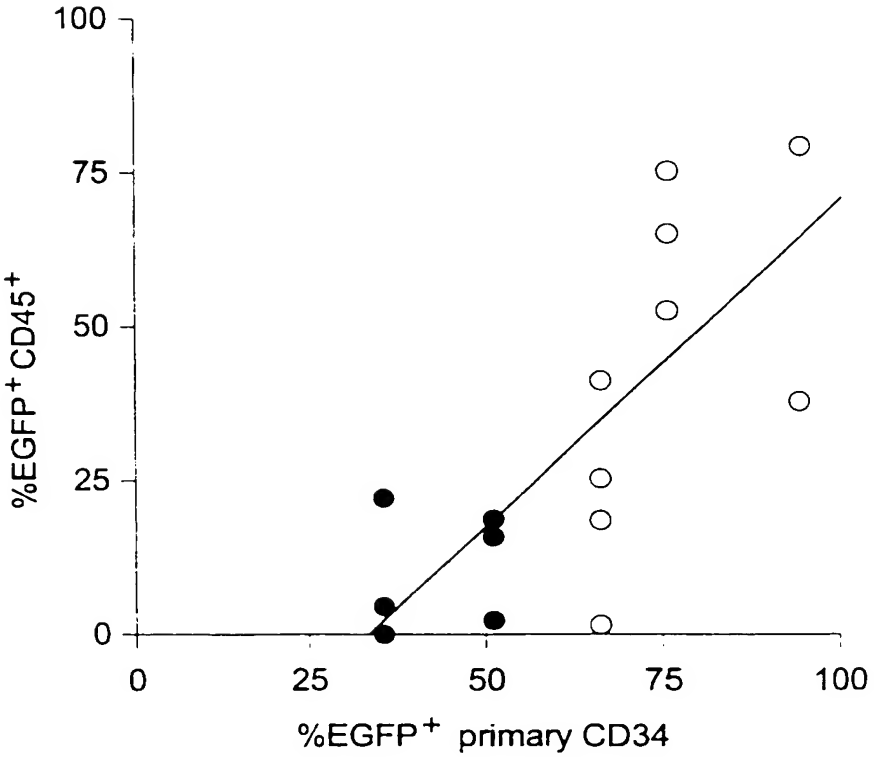
100
75
50
25
0
%EGFP+CD45+
0
25
50
75
100
%EGFP+ primary CD34

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL 00/00611

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/86 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HANENBERG H ET AL: "Optimization of fibronectin-assisted retroviral gene transfer into human CD34+ hematopoietic cells." HUMAN GENE THERAPY, (1997 DEC 10) 8 (18) 2193-206., XP000867308	1-9, 12-17
Y	see the whole document and in particular section "target cell concentration" in p. 2197	11,18-20
	--- -/--	

[X] Further documents are listed in the continuation of box C

[ ] Patent family members are listed in annex

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 January 2001	02/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040 Tx. 31 651 epo nl, Fax (+31-70) 340-3016	ALCONADA RODRIG.., A

4

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Internation plication No
PCT/NL 00/00611

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
Y	KIEM H P ET AL: "Improved gene transfer into baboon marrow repopulating cells using recombinant human fibronectin fragment CH-296 in combination with interleukin-6, stem cell factor, FLT-3 ligand, and megakaryocyte growth and development factor." BLOOD, (1998 SEP 15) 92 (6) 1878-86., XP002128462	11
A	the whole document	1-9, 12-17

Y	HENNEMANN B ET AL: "Optimization of retroviral-mediated gene transfer to human NOD/SCID mouse repopulating cord blood cells through a systematic analysis of protocol variables." EXPERIMENTAL HEMATOLOGY, (1999 MAY) 27 (5) 817-25., XP000867307	11
A	the whole document	1-9, 12-18

Y	BAUER T R ET AL: "Retroviral-mediated gene transfer of the leukocyte integrin CD18 into peripheral blood CD34+ cells derived from a patient with leukocyte adhesion deficiency type 1." BLOOD, (1998 MAR 1) 91 (5) 1520-6., XP002128463	11,18-20
A	the whole document	1-4,6-17

Y	TAKIYAMA ET AL: "Comparison of methods for retroviral mediated transfer of glucocerebrosidase gene to CD34+ hematopoietic progenitor cells" EUROPEAN JOURNAL OF HEMATOLOGY, (1998 JULY) 61 (1) 1-6, XP000867265	18-20
A	the whole document	1-9, 12-17

Y	FREIE B W ET AL: "Correction of Fanconi anemia type C phenotypic abnormalities using a clinically suitable retroviral vector infection protocol." CELL TRANSPLANTATION, (1996 MAY-JUN) 5 (3) 385-93., XP000867303	18-20
A	the whole document	1-9, 12-17

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P50739PC00		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL00/00611	01/09/2000	02/09/1999

International Patent Classification (IPC) or national classification and IPC
C12N15/86

Applicant
ERASMUS UNIVERSITEIT ROTTERDAM et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/04/2001	02.01.2002

Name and mailing address of the international preliminary examining authority:
European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax +49 89 2399 - 4465

Authorized officer
Roscoe, R
Telephone No. +49 89 2399 2554

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00611

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-16 as originally filed

Claims, No.:

1-20 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:
   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☒ all parts.

   ☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-7
	No:	Claims	8-20
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-20

Industrial applicability (IA)	Yes:	Claims	1-20
	No:	Claims	

2. Citations and explanations
see separate sheet

IV. **Lack of Unity**

The present claims contain at least 2 invention groups. For practical reasons, this objection will not be dealt with in the International Phase.

The invention groups are:

1) Claims 1-7 Improved transduction method
2) Claims 8-20 Method for selecting high producer cell lines and uses of viruses obtained therefrom.

V. **Reasoned statement on Novelty, Inventive Step and Industrial Applicability**

The documents mentioned in the present International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc.

- **Novelty (Art.33(2) PCT)**

Claims 1-7 are considered novel since D1 does not explicitly test a batch of CD34+ cells for optimal transduction efficiency and then proceed to use the rest of the batch at the determined best concentration.

Claims 8-11 are anticipated by e.g. D6. Passage spanning p386 and p387 demonstrates the selection of the highest virus-producing cell line.

Claims 12-20 are product-by-process claims or claims dependent thereon or referring thereto. Looking at claim 12, for example, a composition containing a "high" concentration of retrovirus particles can be obtained by various methods. Such a composition is not distinguishable by the process from which it was derived, since in the present case the method used does not impart any properties on the retroviral particles per se.
Large numbers of prior art documents, including all 6 cited documents, disclose compositions with high retrovirus titres and various of these documents (e.g. D5, D6) use CD34+ cells transformed with these viruses in the treatment of disease

conditions. Hence, claims 12-20 are not novel.

- **Inventive Step (Art.33(3) PCT)**

The present application contains absolutely no inventive subject-matter. Applicant merely claims various routine workshop modifications of a known procedure. Optimization of concentrations of various reaction components to achieve maximum yield or selection of the highest producer cell line are all procedures routinely performed by all scientists in the fields of biology or chemistry. Usually these basic optimizations are considered so trivial that they are not even disclosed.

Regarding claims 1-7, D1 already shows optimization of target cell concentration (p2167) and of levels of virus applied (p.2196 plus reference to Paul et al., 1993 etc. on p.2194). Further, also uses fibronectin analogs to enhance binding. It is obvious for a skilled person selecting an efficient protocol for transducing a batch of retroviral delivery particles would optimize his protocol using the optimization procedures of D1 or even simply by using his own common sense in the context of his scientific knowledge.

Regarding selection of the highest producing clone (e.g. claims 8-), this is a common-place procedure and applicant appears to have no embodiments in the application as a whole that could be considered inventive in this context.

- **Industrial Applicability (Art.33(4) PCT)**

The present claims appear to have industrial applicability.

PATENT COOPERATION TREA

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
P50739PC00		
International application No.	International filing date *(day month year)*	(Earliest) Priority Date *(day month year)*
PCT/NL 00/00611	01/09/2000	02/09/1999
Applicant		
ERASMUS UNIVERSITEIT ROTTERDAM et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [ ] as suggested by the applicant.

   [X] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

PATENT COOPERATION TREATY

PCT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

PRINS, A., W.
Vereenigde
~~Nieuwe Parklaan 97~~
NL-2587 BN The Hague
~~PAYS-BAS~~

2-3-2002

ONTVANGEN
10 JAN 2002
AMERSFOORT

rap ct
25.81 c2

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Applicant's or agent's file reference: P50739PC00	Date of mailing (day/month/year): 02.01.2002
	IMPORTANT NOTIFICATION

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/NL00/00611	01/09/2000	02/09/1999

Applicant
ERASMUS UNIVERSITEIT ROTTERDAM et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Zoglauer, H Tel +49 89 2399-8051

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P50739PC00		
International application No	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL00/00611	01/09/2000	02/09/1999
International Patent Classification (IPC) or national classification and IPC		
C12N15/86		
Applicant		
ERASMUS UNIVERSITEIT ROTTERDAM et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
02/04/2001	02.01.2002
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel +49 89 2399 - 0 Tx 523656 epmu d Fax +49 89 2399 - 4465	Authorized officer Roscoe, R Telephone No. +49 89 2399 2554

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00611

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-16 as originally filed

Claims, No.:

1-20 as originally filed

Drawings, sheets:

1/6-6/6 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00611

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-7
	No:	Claims 8-20
Inventive step (IS)	Yes:	Claims
	No:	Claims 1-20

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00611

Industrial applicability (IA) Yes: Claims 1-20
No: Claims

2. Citations and explanations
see separate sheet

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/NL00/00611

IV. Lack of Unity

The present claims contain at least 2 invention groups. For practical reasons, this objection will not be dealt with in the International Phase.

The invention groups are:

1) Claims 1-7 Improved transduction method
2) Claims 8-20 Method for selecting high producer cell lines and uses of viruses obtained therefrom.

V. Reasoned statement on Novelty, Inventive Step and Industrial Applicability

The documents mentioned in the present International Preliminary Examination Report are numbered as in the search report, i.e. D1 corresponds to the first document of the search report etc.

Novelty (Art.33(2) PCT)

Claims 1-7 are considered novel since D1 does not explicitly test a batch of CD34+ cells for optimal transduction efficiency and then proceed to use the rest of the batch at the determined best concentration.

Claims 8-11 are anticipated by e.g. D6. Passage spanning p386 and p387 demonstrates the selection of the highest virus-producing cell line.

Claims 12-20 are product-by-process claims or claims dependent thereon or referring thereto. Looking at claim 12, for example, a composition containing a "high" concentration of retrovirus particles can be obtained by various methods. Such a composition is not distinguishable by the process from which it was derived, since in the present case the method used does not impart any properties on the retroviral particles per se.
Large numbers of prior art documents, including all 6 cited documents, disclose compositions with high retrovirus titres and various of these documents (e.g. D5, D6) use CD34+ cells transformed with these viruses in the treatment of disease

conditions. Hence, claims 12-20 are not novel.

- **Inventive Step (Art.33(3) PCT)**

The present application contains absolutely no inventive subject-matter. Applicant merely claims various routine workshop modifications of a known procedure. Optimization of concentrations of various reaction components to achieve maximum yield or selection of the highest producer cell line are all procedures routinely performed by all scientists in the fields of biology or chemistry. Usually these basic optimizations are considered so trivial that they are not even disclosed.

Regarding claims 1-7, D1 already shows optimization of target cell concentration (p2167) and of levels of virus applied (p.2196 plus reference to Paul et al., 1993 etc. on p.2194). Further, also uses fibronectin analogs to enhance binding. It is obvious for a skilled person selecting an efficient protocol for transducing a batch of retroviral delivery particles would optimize his protocol using the optimization procedures of D1 or even simply by using his own common sense in the context of his scientific knowledge.

Regarding selection of the highest producing clone (e.g. claims 8-), this is a common-place procedure and applicant appears to have no embodiments in the application as a whole that could be considered inventive in this context.

- **Industrial Applicability (Art.33(4) PCT)**

The present claims appear to have industrial applicability.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

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Applicant VERSTEGEN, Monique, Maria, Andrea et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

02 April 2001 (02.04.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Pascal Piriou
Facsimile No. 41 22 740 14.35	Telephone No. 41 22 338 83 38

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